The Role of E2F3 in Bladder Cancer Functions

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation

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by

Jeffrey J. Miller

The Ohio State University

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Project Adivsor: Dr. Kamal Pohar, Department of Urology

Abstract

Bladder cancer is one of the most expensive types of cancer to treat due to its high recurrence rate and the costly methods of its treatment. Improved mechanistic understanding of genetic changes commonly associated with bladder cancer and how the changes contribute to cancer development will lead to the identification of new therapeutic targets. These molecular targets may increase patient survival rate and decrease reoccurrence of tumors. A number of experimental studies support the role of E2F genes in regulating cell cycle progression and gene expression for a broad spectrum of biological functions. Retinoblastoma (pRB), an important tumor suppressor, is a major regulator of E2F genes, and, traditionally, inactivation of RB is sufficient to induce cell proliferation by releasing E2F3, a transcription factor involved in cell cycle progression. However, E2F3 is commonly over-expressed in high-grade and stage tumors. Our hypothesis is that co-existence of pRB inactivation and E2F3 over-expression in the same tumor implicate E2F3 as an integral protein in promoting cancer cell functions beyond cell cycle progression. Using a bladder cancer cell line which has over-expression of E2F3 and loss of pRb, we have knocked down the expression of E2F3 and analyzed the change in specific cellular functions, including proliferation and invasion. We have also developed a mouse model of bladder cancer that is representative of the human condition. We found that upon E2F3 knockdown, cellular invasion levels decreased by about 40%. Although more invasion assays are needed to confirm this data, a drop in cellular invasion upon E2F3 knockdown substantiates our original hypothesis, possibly explaining E2F3 as a major regulator in the molecular pathway of cancer cell invasion.

Introduction

Currently, bladder cancer stands as the 4th most common cancer in males and the 8th most common in females. However, non-surgical bladder cancer treatment is cost-ineffective and not significant in improving patient survival rates (Botteman et al., 2006). Therapeutic treatment often does not prevent the need for surgical treatments, particularly radical cystectomy. This is particularly troubling when one looks at the cost involved in treating bladder cancer and its prevalence. Cost per patient for bladder cancer is the highest of all cancers, primarily because of the high rate of occurrence and the need for life-long monitoring and treatment. Cost of treatment from diagnosis to death, on average, ranges from $96,000 to $187,000 (Botteman et al., 2006).

Cancer research has become increasingly focused on molecular therapeutic targets as a non-surgical treatment option (Gibbs, 2002). With current non-surgical treatment options for bladder cancer being cost-ineffective, molecular targets may be the best way to ensure patient survival and prevent tumor reoccurrence.

E2F3 is a transcription factor involved in cell cycle progression (Humbert et al., 2000). It has been shown that a critical threshold level of certain E2F3-regulated genes determines the timing of the G1/S cell cycle transition, and thereby the rate of cellular proliferation in normal cells (Leone et al., 1998). E2F3 exists as two isoforms (E2F3a and E2F3b) and the gene is located on chromosome 6p22.3 (Chong et al., 2009). The majority of our knowledge of E2F3 is from cell lines and more recently animal models of development (Tsai et al., 2008).

Whether E2F family members and specifically E2F3 have a role in cancer is not well studied. E2F3 may play a role in oncogenic functions as several malignancies have increased expression of the protein (Hurst et al., 2008). Bladder cancer is probably the best example as 30-

40% of human tumors have increased expression. Members of the E2F family, including E2F3a and E2F3b, are key targets of the tumor suppressor protein, retinoblastoma (Weinberg, 1995). The retinoblastoma protein (pRB) is the major regulator of these transcription factors and this function is intimately connected with its tumor suppressor function. Phosphorylation of pRB leads to the release of the E2F3 protein and the subsequent transcription of cell cycle-initiating genes such as B-myb, cyclin A,cdc2, cdc6, and DHFR. As is the case in other human tumors, pRB is often inactivated in bladder cancer (Horowitz et al., 1990). This would suggest E2F3 is free to execute its proliferative function in the majority of bladder tumors; however studies in bladder cancer cell lines and human tumors demonstrate that increased expression of E2F3 is often due to gene amplification (Feber et al., 2004).

In our lab, over 130 human bladder tumors were examined by immunohistochemistry (IHC) and the majority of these tumors (84%) had RB inactivation, regardless of tumor grade or stage. It was found that E2F3 was over-expressed in 38% of the bladder tumors that had RB inactivation. Importantly, in over 90 % of the tumors that had increased expression of E2F3, it was on the basis of gene amplification or gain of function in chromosome 6p22.3 as determined by fluorescence in situ hybridization (FISH). The classic paradigm suggests that inactivation of pRB releases E2F3 and would be sufficient to enhance cell proliferation; it was interesting to examine why the human bladder tumors studied had E2F3 gene amplification with inactivated pRB simultaneously. As a translational research lab, we were less interested in the mechanisms behind the occurrence of these co-existing events than in the consequences of said genetic events. What functions do bladder cancer cells gain when pRB inactivation and E2F3 gene amplification are coexisting events in the same tumor? The hypothesis from these observations

is that E2F3 may have non-cell-cycle dependent functions which facilitate bladder tumor progression (i.e., invasion or differentiation).

To study the effects of this molecular relationship, a bladder cancer cell line with loss of RB and E2F3 gene amplification was used. To specifically examine the role of E2F3, our lab created a stable cell line with E2F3 knockdown. Both the E2F3a and E2F3b isomers in this cell line were knocked down by shRNA after transduction by lentivirus. Knockdown levels of E2F3 protein were confirmed to be approximately 60% by Western blotting. And E2F3 mRNA levels were confirmed to be knocked down approximately 60% as well by qPCR.

The mRNA expression levels between the stable E2F3-knockdown cell line and a control line were compared via microarray analysis. A biostatistician found significant changes in mRNA expression levels for genes (CDC25A, FOX01, and others) which are traditionally correlated with increased cellular proliferation in the literature (Adachi et al., 2007) (Ray et al., 2007). This supported the traditional role of E2F3 as a regulator of cell cycle progression and subsequent proliferation. The same biostatistician also confirmed significant decreases in mRNA expression levels of PTGS2 (or COX-2) which has been linked in the literature with invasive qualities of breast cancer cells (Ray et al., 2007). This microarray data led to the decision to perform functional assays on proliferative and invasive qualities of E2F3-knockdown cell lines.

To study the effects of E2F3 knockout *in vivo*, we have created a mouse model with RB inactivation and E2F3a overexpression which mimics the genetic events found in human tumors. Mice were used as a model because E2F3 is conserved in mammals, as well as their physiological similarities to humans. Animal models are useful to understanding the mechanisms of cancer and testing new treatments. By developing animal models with genetic

events similar to the human conditions, we will better understand bladder cancer mechanisms and likely develop new therapeutic targets for bladder cancer.

Our goal with this project is to implicate E2F3 as a potential target for molecular therapy in bladder cancer treatment. We expect E2F3 over-expression to correlate with increased invasive and proliferative qualities in bladder cancer cell lines. And we expect E2F3 over-expression in mouse models to show unique tumor phenotypes similar to human patients we have seen.

Materials and Methods

Mouse Model

To study the consequences of pRB inactivation and E2F3a over-expression events in bladder epithelia a novel quadruple-transgenic mouse line was constructed. These genetic events were controlled spatially by using uroplakin II (UPII), a promoter specific to bladder epithelia. To accomplish knockout of pRB, a Cre-Lox recombination system was used with an $RB^{f/f}$ mouse kindly provided by Dr. Gustavo Leone's laboratory and a UPII-Cre mouse kindly provided by Dr. Xue-Ru Wu's laboratory. These mice were crossed to knockout pRB. To accomplish over-expression of the E2F3a gene, a Tet-On system was used with a tetO-E2F3a positive mouse and a UPII-rtTA positive mouse which were constructed by Dr. Pohar's lab before my involvement with the project. These mice were crossed to accomplish over-expression of E2F3. These two mouse strains were crossed to acquire mice with both pRB inactivation and E2F3 over-expression (See Figure 1). Induction of E2F3a over-expression *in vivo* was controlled temporally by adding doxycyline to the mouse's diet. Doxycycline is a tetracycline derivative, which allows binding of rtTA to the operator upstream of the E2F3 constructed gene, allowing over-expression of E2F3. Doxycycline (1 g/Kg) was fed to the mice immediately after determining their genotype, which occurred approximately a month after birth for most mice.

All mouse bladder tissue samples were dissected and placed in formalin. After 2 days they were processed by the Ohio State Histology Lab and returned to our lab as microscope slides.

DNA Isolation and PCR

Mouse genotypes were determined via DNA isolation and subsequent PCR reactions. Standardized protocols were used for both experiments. Primers for the four constructed genes were developed and ordered from Invitrogen before my involvement with the project.

Cancer Cell Functional Assays

To evaluate the impact of E2F3 on cancer cell functions, assays were performed on HT1376, a human bladder cancer cell line with E2F3 gene amplification and pRB inactivation, which was purchased from ATCC. Assays results were compared between a control HT1376 line and a stable E2F3-knockdown HT1376 line. Media used for the following experiments was DMEM Dulbecco's Modified Eagle Media High Glucose 1X (Gibco) and Opti-MEM Reduced Serum Medium 1X (Invitrogen). Plasmids for the BrdU experiment included RSM7, a mutated isoform of pRB which cannot be phosphorylated, and WT RB, an isoform of pRB which can be freely phosphorylated, were kindly provided by Dr. Gusatavo Leone's laboratory.

BrdU Assay

A proliferation assay was conducted on both the E2F3-knockdown cell line and the control cell line. Protocol for this assay was kindly provided by Dr. Gustavo Leone's laboratory. The proliferation assay measures cellular proliferation through incorporating BrdU into the cellular environment. Cells that have proliferated since introduction of BrdU are stained pink and can be counted individually. Three experiments were run. In experiment 1), only the difference between the E2F3-knockdown cell line and control cell line without introduction of pRB was analyzed. In experiment 2), RSM7 was used to constitutively reintroduce

dephosphorylated, or the active, isoform of RB in both the E2F3-knockdown cell line and the control cell line. In experiment 3), wild-type RB, which can be freely phosphorylated, was reintroduced to both cell lines. This wild-type RB acted as the control group.

An 8-well culture slide was used to perform the BrdU assay. Three wells were used for the E2F3-knockdown cell line and three wells were used for the control line (See Figure 2). Approximately 700 cells from the respective cell lines were added to six of these 0.7 cm^2 wells and allowed to incubate for 24 hours. Cells were than transfected with DNA plasmids combined with Lipofectamine, from Invitrogen. In experiment 1), cells were transfected with no plasmids, but 1.5 µL of Lipofectamine and 150 µL Opti-MEM media were added to the two wells. In experiment 2), cells were transfected with 0.6 µg of RMS7-LP plasmid combined with 1.5 µL Lipofectamine and 150 µL Opti-MEM media. In experiment 3), cells were transfected with 0.6 µg WT-LP plasmid combined with 1.5 µL Lipofectamine and 150 µL Opti-MEM media. Cells were then allowed to incubate for 48 hours.

At the end of this incubation period, a working solution of BrdU was created by diluting 10 µL of a stock 10mM BrdU solution with 990 µL PBS. A working solution of BrdU in the amount of 30 µL was added to each well and the cells were allowed to incubate for 1.5 hours. Cells were then:

- washed with PBS twice
- fixed at room temperature with 1:1 methanol:acetone for 20 minutes
- washed with PBS twice
- treated with 1.5 M HCl for 30 minutes
- washed with PBS twice
- washed with PBS/0.5% BSA once

- blocked with PBS/0.5% BSA for 10 minutes (Performed in humid chamber)
- stained with primary antibody for 2 hours. Primary antibody was created by diluting α-BrdU antibody (BrdU Ab-3 from Neomarkers) 1:100 in PBS and adding 1:1000 DNAase and 1:25 $MgCl_2$ (25mM). (Performed in humid chamber)
- washed with PBS twice
- washed with PBS/0.5% BSA once
- blocked with PBS/0.5% BSA for 10 minutes (Performed in humid chamber)
- stained with secondary antibody for 1.25 hours. Secondary antibody was created by diluting Alexa-flour α-mouse antibody (Alexa-Fluor 568 from Invitrogen) 1:75 in PBS. (Performed in humid chamber in the dark)
- washed with PBS thrice (Performed in dark)
- stained with DAPI for 1.25 hours. DAPI solution was created by diluting DAPI 1:250 in PBS. (Performed in humid chamber in the dark)
- washed with PBS twice (Performed in dark)
- mounted with Immu-mount onto microscope slides
- analyzed via fluorescent microscopy

Invasion Assay

An invasion assay was also conducted on the E2F3-knockdown cell line and the control cell line. Inserts for this assay were BD Biocoat Matrigel Invasion Chambers. Protocol for this assay also came from the Invasion Chamber kit. Cells were placed in a solution of high glucose media with 0% FBS and incubated in an insert surrounded by a well that contained high glucose media with 10% FBS. Cells with invasive qualities released proteins that degraded the Matrigel

layer and created holes whereby they invaded to the chemo attractant layer of the insert (See Figure 3).

A cell concentration of 2×10^5 cells/mL was added to the inserts. The invasion chambers were allowed to incubate for 22 hours. Afterwards, cells were fixed and stained. Cellular invasion numbers were then analyzed via microscopy.

Statistics

All p values of cell numbers were calculated using t tests on Microsoft Excel. Statistical significance was defined as a p value less than 0.05.

Results

Mouse Model

A transgenic mouse model of inactivation of pRB and increased expression of E2F3 was generated over the course of a year and a half. Total, both alive and already dissected, 32 mice with this specific genotype have been generated. Of the mice still alive, six mice have been fed with doxycycline for six months or more and eight mice have been fed for three months or more. Control mice have also been generated with the following genotypes: i) wild-type, ii) inactivation of pRB and iii) over-expression of E2F3. Of the mice still alive, there are i) 19 wild-type mice, ii) seven inactivation of pRB, and iii) eleven E2F3 over-expression mice that have been fed doxycycline (See Figure 4).

Three hours after the death of an inactivation of pRB and increased expression of E2F3 mouse, its bladder was dissected and reviewed via paraffin slides. This mouse had been fed doxycycline for approximately six months. H&E staining of the tissue was confirmed by a pathologist to show signs of papillary carcinoma. The tissue was then stained for E2F3 and Ki67, a common marker for cell-cycle progression. Ki67 can be used to detect cell-cycle progression because it is only found in active stages of the cell cycle, and is absent from resting cells. E2F3 and Ki67 signals were concentrated in the region of papillary carcinoma and were prominent in similar locations on the tissue (See Figure 5).

Six UPII-Cre; RB $^{f/f}$; teto-E2F3a; UPII-rtTA mice, not including the mouse that exhibited signs of papillary carcinoma, were dissected and their bladder tissue placed on microscopic slides. All six of these mice have been fed doxycycline for at least six months; however these mice have not been analyzed by a pathologist or subjected to immunohistochemical stains.

BrdU Assay

Pictures were taken of large quantities of cells and counted for evidence of proliferation (See Figure 6a). Fields of cells were chosen at random, and approximately 6 to 8 fields were counted for each well of cells. This experiment was not blinded, as I counted cells for evidence of proliferation and also knew which cells were controls and which were experimental. A minimum of 500 cells were counted total for each well, excluding experiment 3 which did not have 500 cells in the wells (See Figure 6b).

In experiment 1), it was found that in the control cell line, 445 cells showed evidence of proliferation and 463 cells showed evidence of no proliferation. The ratio of proliferating cells was 0.96:1 and the p value of this set was 0.762. It was found in the E2F3-knockdown cell line that 423 cells showed evidence of proliferation and 610 cells showed no evidence of proliferation. The ratio of proliferating cells was 0.69:1 and the p value of this set was 0.017.

In experiment 2), it was found that in the control cell line, 292 cells showed evidence of proliferation and 462 cells showed evidence of no proliferation. The ratio of proliferating cells was 0.63:1 and the p value of this set was 0.008. It was found in the E2F3-knockdown cell line that 271 cells showed evidence of proliferation and 511 cells showed no evidence of proliferation. The ratio of proliferating cells was 0.53:1 and the p value of this set was 0.004.

In experiment 3), it was found that in the control cell line, 407 cells showed evidence of proliferation and 518 cells showed evidence of no proliferation. The ratio of proliferating cells was 0.78:1 and the p value of this set was 0.090. It was found in the E2F3-knockdown cell line that 179 cells showed evidence of proliferation and 196 cells showed no evidence of proliferation. The ratio of proliferating cells was 0.91:1 and the p value of this set was 0.492.

Invasion Assay

In the control cell line, it was found that 659 cells had invaded past the Matrigel membrane into the chemo attractant layer compared to 312 cells from the E2F3-knockdown line (See Appendix 14). The p value for this set was 0.0008. A replicate experiment was run independently of the first invasion assay. In this replicate experiment, it was found that 233 cells had invaded from the contol cell line and 144 cells had invaded from the E2F3-knockdown cell line. The p value for this set was 0.476.

Discussion

Mouse Model

At this point in time, mouse model data is insufficient and no significant conclusions can be drawn from the one mouse that has been analyzed. Although one mouse with pRB inactivation and E2F3 over-expression shows evidence of papillary carcinoma, the sample size is not large enough to confirm statistical significance. Currently, there are six mouse bladder tissue samples with inactivation of pRB and over-expression of E2F3 that are waiting to be analyzed by a pathologist. I analyzed these slides and found evidence of hyperplasia in one mouse. When these tissue samples, and their controls, have been analyzed by a pathologist, correlations can be drawn between their specific genotype and histological data. These tissues will also need to be stained for genes shown in the literature to be important in cancer cell proliferation and invasion pathways such as Ki67 and COX-2 respectively.

Current numbers of mice with pRB inactivation and E2F3 over-expression will be fed doxycycline until it is deemed necessary to examine their bladder phenotype. Significant time points would be six months and one year.

BrdU Assay

In experiment 1), a significant correlation between E2F3 knockdown and decreased cellular proliferation was found ($p<0.05$). After E2F3 knockdown, cellular proliferation decreased by approximately 13.5%, which supports the traditional view of E2F3 as an important agent in cellular proliferation pathways. In experiment 2), a significant distinction between proliferation levels of cells was found between cells which had RMS7 introduced and cells which had pRB inactivated ($p<0.05$). Cellular proliferation decreased by approximately 15%

when a constitutively, activated form of RB was reintroduced to cells. Similar to experiment 1), there was a decrease in cellular proliferation in the E2F3-knockdown line when compared with the control line. However, cellular proliferation in experiment 2) was decreased in both the stable knockdown and control cell lines by approximately 15% when compared with experiment 1).

Results for experiment 3) were more difficult to interpret. Cells were clumped together and it was difficult to distinguish individual cells among these clumps. Staining was also not as strong, and it was difficult, subsequently, to determine if cells had the BrdU marker. In experiment 3), there was an increase in cellular proliferation upon E2F3-knockdown. Those data do not correlate with previous experiments and may be attributed to procedural errors.

Invasion Assay

A significant correlation between E2F3 knockdown and decreased cellular invasion was found ($p<0.05$). Invasive qualities of cells decreased by 53% after E2F3 knockdown, and this number was supported by a replicate experiment where cellular invasion decreased by 40% after E2F3 knockdown. These experiments indicate that E2F3 is correlated with invasive qualities of bladder cancer cells. This supports our original hypothesis that E2F3 is involved in cancer functions beyond cell cycle progression and cellular proliferation.

However, data from the replicate experiment was not statistically significant with a p value of 0.476. This could be explained by the amount of cells counted (377 instead of 971 in the first experiment) which would lower the N value of the experiment leading to a larger p value. Future experiments will need to be conducted to gain a consistent idea of the relative number of invasive cells upon E2F3 knockdown.

Limitations

It is important to note that the proliferation assay was only performed once with no replicate experiments. Similarly, the invasion assay was run only twice. The correlation between decreased levels of invasion or proliferation and E2F3 knockdown would be a more convincing argument if replicate assays were performed.

Another limitation is a lack of data on direct targets of E2F3. Although decreases in proliferative and invasive cell qualities can be correlated with E2F3-knockdown, it cannot be confirmed that E2F3 directly initiates these pathways. To confirm that E2F3 is a causal agent in invasive pathways, ChIP-Sequencing data will need to be analyzed. ChIP-Sequencing data will identify direct binding targets of the E2F3 protein and allow confirmation that E2F3 is a causal agent in the invasion pathway of bladder cancer cells.

Conclusion and Further Directions

These experiments indicate that E2F3 over-expression may facilitate bladder tumor functions beyond cell cycle progression and cellular proliferation. Specifically, E2F3 over-expression may be correlated with bladder cancer cell invasion. This correlation is supported by microarray analysis that indicates E2F3 knockdown is also correlated with decreased expression of COX-2, which has been linked with invasive qualities of breast cancer cells. These microarray data will need to be supported with ChIP-Sequencing data that is currently being analyzed, which will identify gene promoters bound by E2F3.

Invasion assays with E2F3 knockdown will continue to be conducted to determine the significance of the first experiment. Invasion assays will also be conducted in the future with

mitomycin, an inhibitor of cellular proliferation, to determine the exact number of invasive cells upon E2F3 knockdown.

In terms of the invasion assay, similar experiments to the ones performed for this thesis will need to be performed with COX-2 inhibitors. Because COX-2 is associated with invasive qualities of cells, introducing an inhibitor to the experiment will allow confirmation that COX-2 is a downstream target of E2F3. If COX-2 is downstream of E2F3, we would be expected to see no change in cellular invasion level in the stable E2F3-knockdown cell line after introduction of COX-2 inhibitor. However, we would expect to see a decrease in invasive qualities in the control cell line. Performing this assay with COX-2 inhibitor and supporting it with Chip-Sequencing data would allow confirmation of COX-2 expression, and a subsequent increase in cellular invasion qualities, as a direct consequence of E2F3 over-expression.

Figures

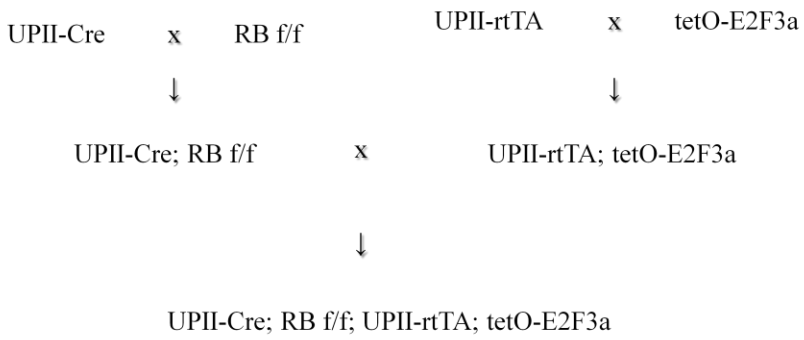


Figure 1

A combination of the Cre-lox recombinase strategy and the rtTA-tetO strategy was used to develop the transgenic mouse model with similar genetic mutations as human bladder tumors that have been analyzed. Uroplakin II is a bladder tissue specific promoter. E2F3a over-expression was induced upon doxycycline introduction to mice.

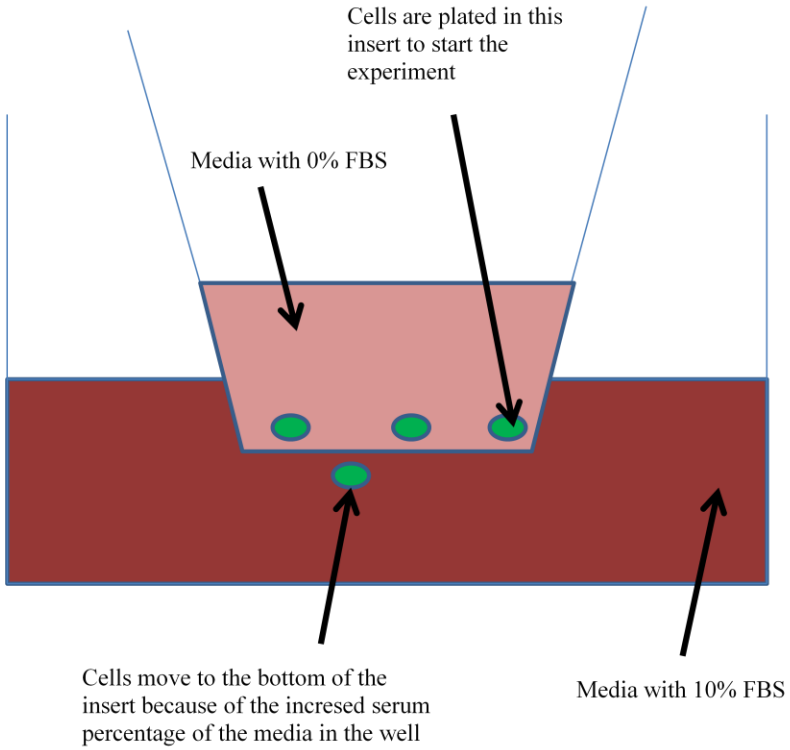
HT1376 with E2F3 Knockdown	1)No reintroduction of Rb protein	2) Reintroduction of consitutively activated Rb	3) Reintroduction of wild-type Rb
HT1376 with no E2F3 Knockdown	1)No reintroduction of Rb protein	2) Reintroduction of consitutively activated Rb	3) Reintroduction of wild-type Rb

Figure 2

Schematic of the proliferation assay experiment. Each box indicates a separate well in the culture plate.

Figure 3

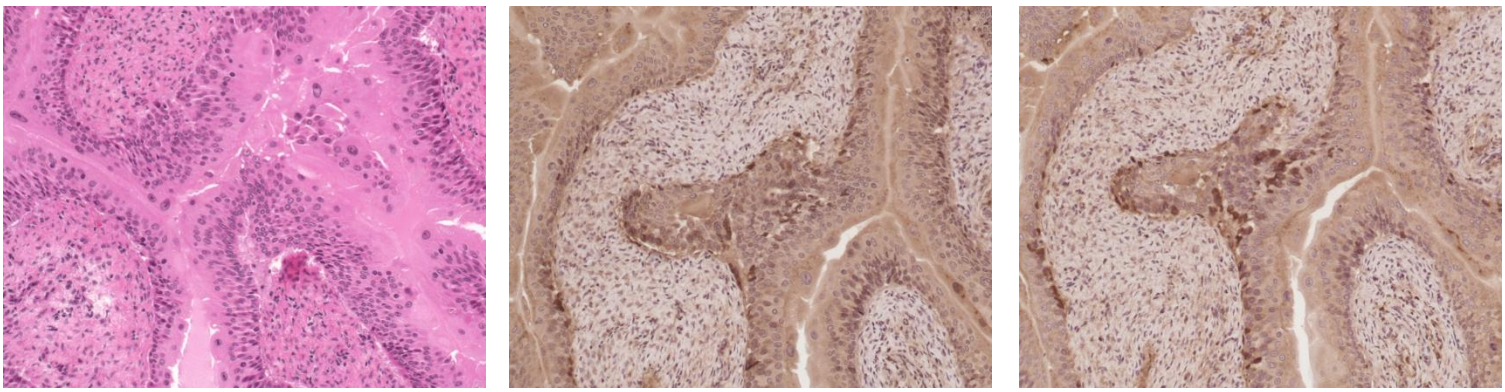
Schematic of the invasion assay experiment.



	Dissected	Alive	Total Mice
UPII-rtTa; tetO-E2F3a	**2**	**7**	**9**
UPII-Cre; RB f/f	**2**	**11**	**13**
UPII-rtT; tetO-E2F3a; UPII-Cre; RB f/f	**7**	**25**	**32**
WT	**4**	**19**	**23**

Figure 4

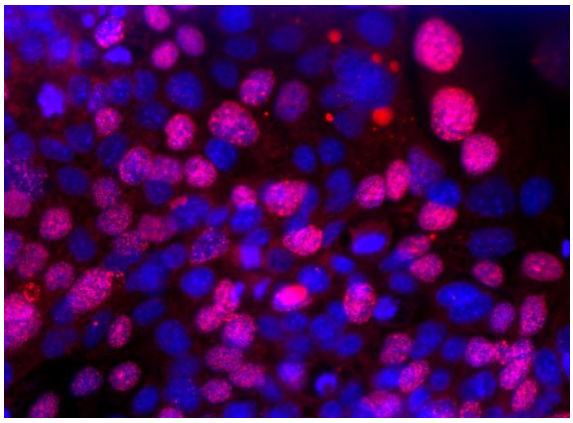
This chart details the total number of mice that have been generated. It also details the genotypes of mice with RB inactivation and E2F3 over-expression and the various controls of the mouse model experiment.



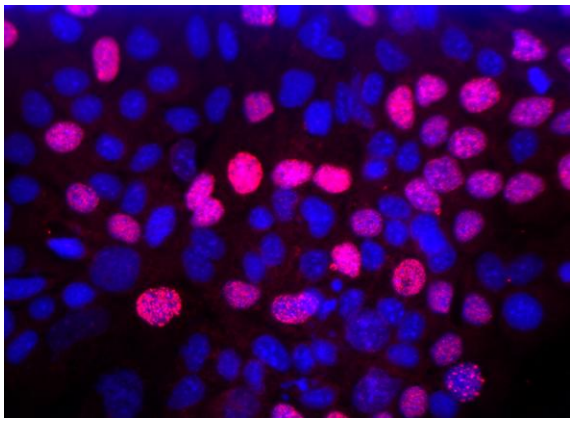
A **B** **C**

Figure 5

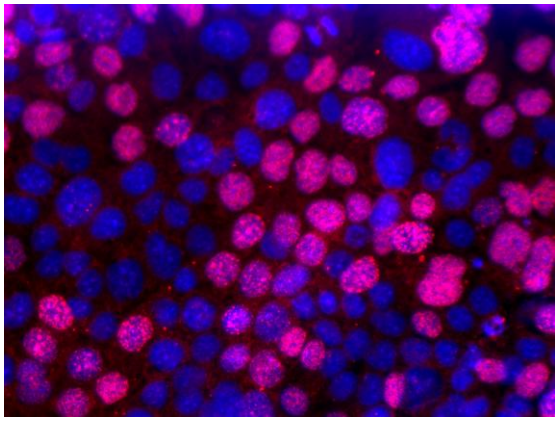
A) H&E staining of UPII-Cre; RB $^{f/f}$; teto-E2F3a; UPII-rtTA mouse. Physiology is indicative of papillary carcinoma. B) Immunohistochemical staining for E2F3 of UPII-Cre; RB $^{f/f}$; teto-E2F3a; UPII-rtTA mouse. C) Immunohistochemical staining for Ki67 of UPII-Cre; RB $^{f/f}$; teto-E2F3a; UPII-rtTA mouse. Note how E2F3 and Ki67 brown staining are in similar locations on the bladder tissue.



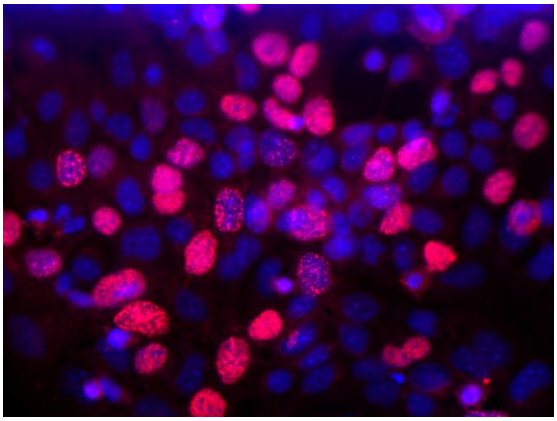
HT 1376 control line (Experiment 1)



HT 1376 line with E2F3 Knockdown (Experiment 1)



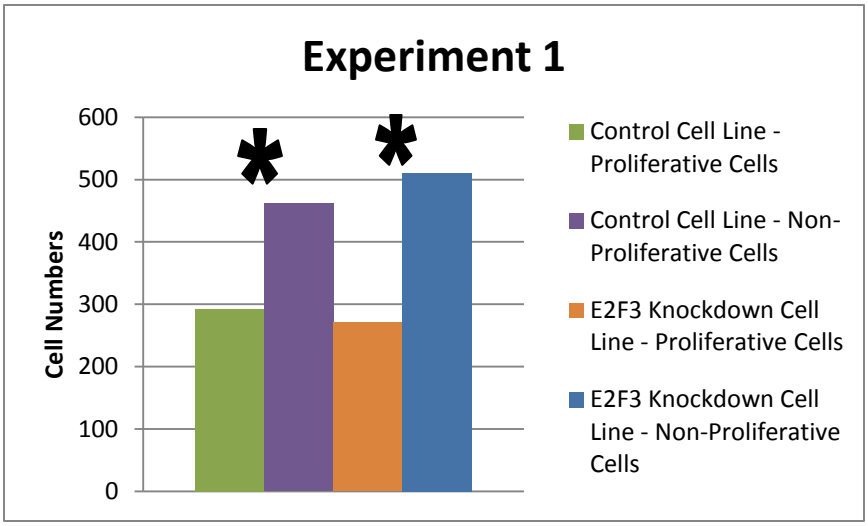
HT 1376 control line w/RMS7 Reintroduction (Experiment 2)

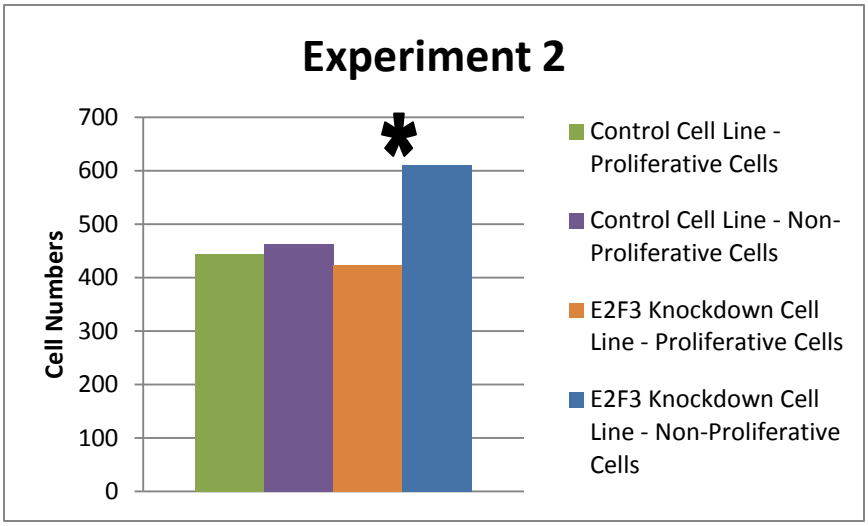


HT 1376 line with E2F3 Knockdown w/RMS7 reintroduction (Experiment 2)

Figure 6a

Cells after BrdU treatment. Pink cells have undergone proliferation since the introduction of BrdU to the cellular environment. Pictures taken at 20x fluorescent microscopy.





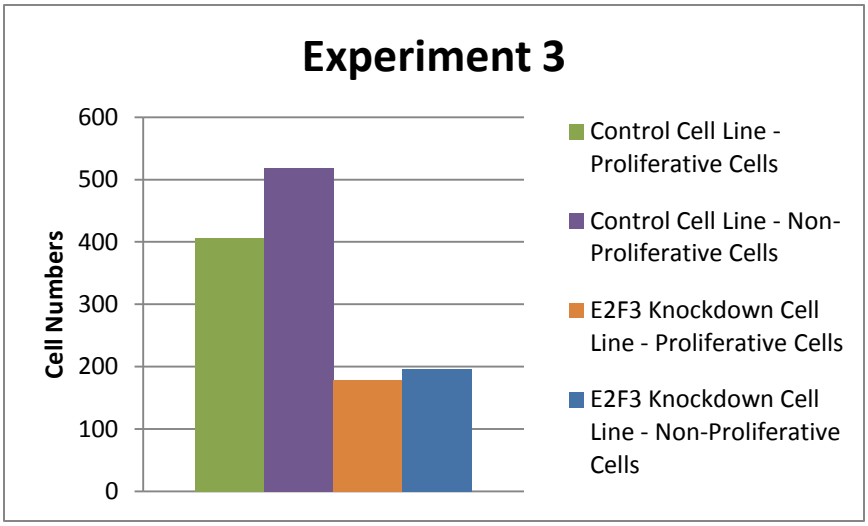
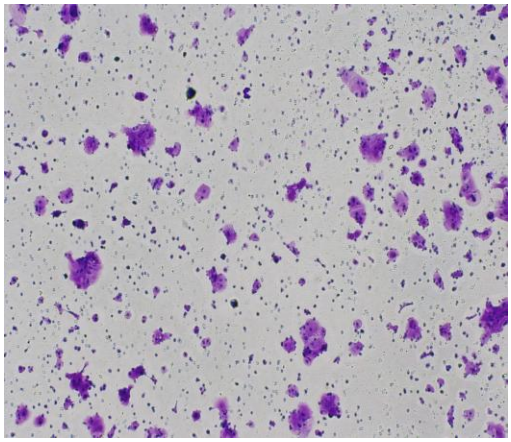
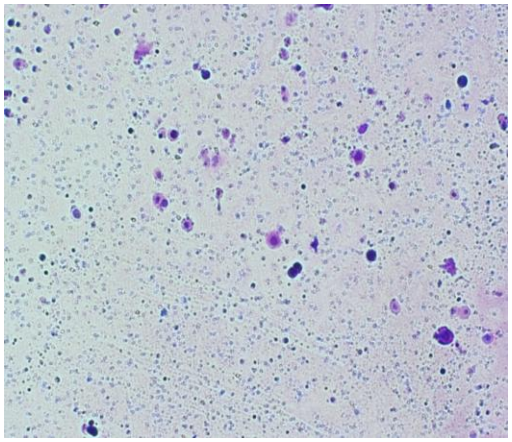


Figure 6b

Charts showing relative cell numbers between proliferative cells and non-proliferative cells. Charts made with Microsoft Office.



HT 1376 control line

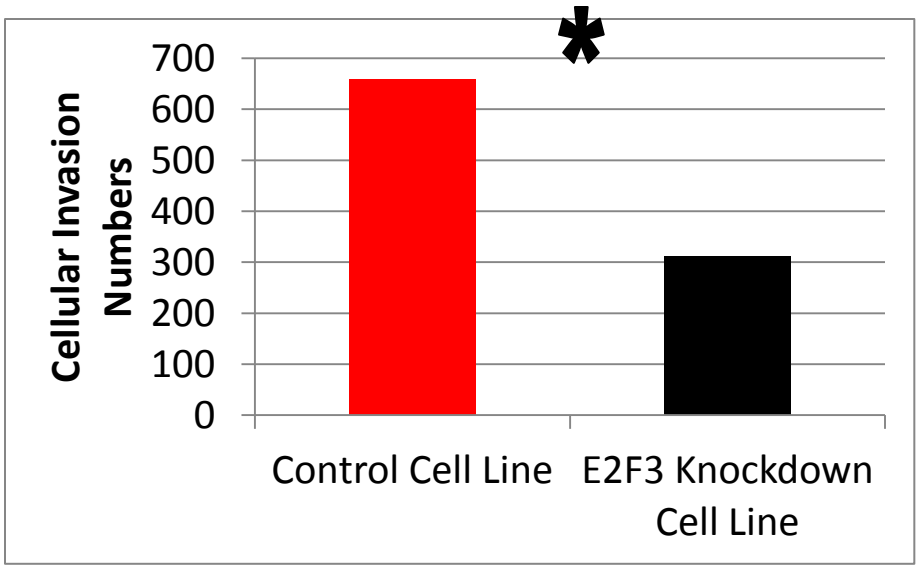


HT 1376 line with E2F3 Knockdown

Figure 7

Invasive cells are stained purple. Pictures taken at 10x microscopy.

Chart showing actual cell numbers for cells which have invaded through the Matrigel. Created in Microsoft Office.



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References

Adachi, M., Osawa, Y., Uchinami, H., Kitamura, T., Accili, D., & Brenner, D. (2007). The Forkhead Transcription Factor FoxO1 Regulates Proliferation and Transdifferentiation of Hepatic Stellate Cells. *Gastroenterology*, **132** (4), 1434-446.

Botteman, M. F., Pashos, C. L., Redaelli A., Laskin, B., & Hauser, R. (2003). The Health Economics of Bladder Cancer. *PharmacoEconomics*, **22** (18), 1315-330.

Chong, J.-L., Tsai, S.-Y., Sharma, N., Opavsky, R., Price, R., Wu, L., et al. (2009). E2f3a and E2f3b Contribute to the Control of Cell Proliferation and Mouse Development. *Molecular and Cellular Biology*, **29** (2), 414-424.

Feber, A., Clark, J., Goodwin, G., Dodson, A. R., Smith, P. H., Fletcher, A., et al. (2004). Amplification and Overexpression of E2F3 in human bladder cancer. *Oncogene,* **23**, 1627-1630.

Gibbs, J. (2000). Mechanism-Based Target Identification and Drug Discovery in Cancer Research. *Science*, **287** (5460), 1969-1973.

Horowitz, J., Park, S., Bogenmann, E., Cheng, J., Yandell, D., Kaye, F., et al. (1990). Frequent Inactivation of the Retinoblastoma Anti-Oncogene is Restricted to a Subset of Human Tumor Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2775-2779.

Humbert, P. O., Verona, R., Trimarchi, J. M., Rogers, C., Dandapani, S., & Lees, J. A. (2000). E2F3 is Critical for Normal Cellular Proliferation. *Genes and Development*. **14**, 690-703.

Hurst, C., Tomlinson, D., Williams, S., Platt, F., & Knowles, M. (2008). Inactivation of the Rb Pathway and Overexpression of Both Isoforms of E2F3 are Obligate Events in Bladder Tumours with 6p22 Amplification. *Oncogene,* **27**, 2716-2727.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Seiichi, I., Williams, R. S., et al. (1998). E2F3 Activity is Regulated During the Cell Cycle and is Required for the Induction of S Phase. *Genes and Development*, **12**, 2120-2130.

Lucci, A., Singh, B., Berry, J. A., Shoher, A., & Ramakrishnan, V. (2004). Cox-2 Overexpression Increases Motility and Invasion of Breast Cancer Cells. *Annals of Surgical Oncology*, **26** (5), 1393-1399.

Ray, D., & Kiyokawa, H. (2007). CDC25A Levels Determine the Balance of Proliferation and Checkpoint Response. *Cell Cycle*, **6** (24), 3039-042.

Tsai, S.-Y., Opavsky, R., Sharma, N., Wu, L., Naidu, S., Nolan, E., et al. (2008). Mouse Development with a Single E2F Activator. *Nature*, **454**, 1137-1141.

Utomo, A. R., Nikitin, A. Y., & Lee, W.-H. (1999). Temporal, Spatial, and Cell Type-Specific Control of Cre-Mediated DNA Recombination in Transgenic Mice. *Nature Biotechnology*, **17**, 1091-1096.

Weinberg, R. (1995). The Retinoblastoma Protein and Cell Cycle Control. *Cell*, **81**, 323-330.